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Tamm-Horsfall protein knockout mice are more prone to urinary tract infection

Rapid Communication

James M. Bates, Jr., Haja Mohideen Raffi, Krishna Prasadan, Ranjan Mascarenhas, Zoltan Laszik, Nobuyo Maeda, Scott J. Hultgren, and Satish Kumar

Department of Medicine and Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, North Carolina; and Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri

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Background. Human colon contains many bacteria that commonly colonize the perineum and frequently enter the urinary tract. Uropathogenic *Escherichia coli* are the most common cause of urinary tract infection. Type 1 fimbriated *E. coli* have been associated with cystitis, and P fimbriated *E. coli* with pyelonephritis. Factors involved in clearing bacteria from the urinary tract are poorly understood. Tamm-Horsfall protein (THP), the most abundant protein in mammalian urine, has been postulated to play a role in defense against urinary tract infection but definitive proof for this idea has been lacking.

Methods. In this study, we generated *THP* gene knockout mice by the technique of homologous recombination, and examined if the THP-deficient ($THP^{-/-}$) mice were more prone to urinary tract infection. Various strains of *E. coli* expressing type 1 or P fimbriae were introduced transurethrally into the bladders of the $THP^{-/-}$ and genetically similar wild-type ($THP^{+/+}$) mice. Urine, bladder, and kidney tissues were obtained from the mice and cultured for bacterial growth.

Results. $THP^{-/-}$ mice inoculated with type 1 fimbriated *E. coli* had a longer duration of bacteriuria, and more intense colonization of the urinary bladder in comparison with $THP^{+/+}$ mice. When inoculated with a P fimbriated strain of *E. coli,* the $THP^{-/-}$ mice showed no difference in kidney bacterial load when compared with the $THP^{+/+}$ mice.

Conclusion. These findings support the idea that THP serves as a soluble receptor for type 1 fimbriated *E. coli* and helps eliminate bacteria from the urinary tract.

Urinary tract infections are common, especially in women [1]. Most urinary tract infections are caused by bacteria that normally reside in the colon, commonly colonize the perineal region, and frequently enter the adjacent urethra [2]. *Escherichia coli* account for 85% of all urinary tract infections [1, 3]. The uropathogenicity of *E. coli* is critically dependent on filamentous appendages on the bacterial surface, called pili or fimbriae. A number of fimbrial types, including type 1, P, S, and Dr adhesions, have been described [3]. Among these type 1 fimbriae have been found to be associated with cystitis, and P fimbriae with pyelonephritis. It is postulated that urine contains host defense factors that continually clear the urinary tract of contaminating bacteria. Currently, these defense mechanisms are not understood fully [3, 4]. Tamm-Horsfall protein (THP) is the most abundant protein in normal urine [5–9]. THP is a polymeric glycoprotein with a monomeric molecular weight of 90,000 Da. It is richly decorated with the most varied array of N-linked, complex type, oligosaccharides found in any human glycoprotein [10]. In addition, some high-mannose [11], and O-linked residues [12] are also present. THP is conserved in evolution [13], and synthesized only in the kidney [14]. The normal physiological function of THP has remained elusive despite extensive study. The biochemical properties of THP and its abundant presence in urine make it a candidate for being a host defense factor that might be involved in clearing bacteria from the urinary tract. Indeed, in vitro studies have shown the ability of THP to bind *E. coli* [15–18], bacteria that commonly cause urinary tract infaction.

In this study, we examined the role of THP as a host defense mechanism against urinary tract infection by creating a mouse deficient in THP by the technique of gene targeting [19], and by determining if the THP-deficient ($THP^{-/-}$) mouse had difficulty clearing bacteria introduced into its bladder.

Key words: Tamm-Horsfall protein, uromodulin, uromucoid, urinary tract infection.

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METHODS

Generation of THP knockout mice

Human THP cDNA probes [14] were used to isolate mouse THP cDNA [20] that was used to probe a mouse

129/sv liver λ Fix II genomic library (Stratagene, LaJolla, CA, USA) to obtain the full-length mouse *THP* gene. The *THP* gene was characterized by restriction analysis, partial sequencing, and comparison with human and rat gene restriction maps [21]. An omega-type replacement targeting vector [22] was constructed incorporating a 2 kb segment 5′ of the cap site of the *THP* gene and the first four exons plus the intervening introns of the *THP* gene (Fig. 1A). The linearized vector was electroporated into embryonic stem cells, obtained from mouse strain 129/sv (brown coat). Successfully transfected embryonal stem cells were injected into developing blastocysts from C57Bl/6 (black coat) mice to obtain chimeric mice. Chimeric mice were bred with Black Swiss female mice and screened by polymerase chain reaction (PCR) of tail DNA to obtain mice heterozygous for THP deficiency. The heterozygous mice were bred with each other to obtain $THP^{+/+}$ and $THP^{-/-}$ offspring. The absence of THP was confirmed by Northern blot (Fig. 1B), Western blot (Fig. 1C), and by immunocytochemistry (Fig. 1D).

The THP mutation was also backcrossed onto the 129/sv strain for seven generations to produce $THP^{+/+}$ and $THP^{-/-}$ offspring with a similar genetic background. The $THP^{-/-}$ mice revealed no gross anomalies. They grew and bred normally. Serum electrolytes, urinalysis, and kidney histology was normal. There was no evidence of pyelonephritis.

Northern blot of mouse kidney for THP RNA

Total RNA was isolated from mouse kidneys by a commercial reagent based on the original method of Chomczynski and Sacchi (TRIzol) (Life Technologies, Rockville, MD, USA) [23]. Fifteen micrograms of total RNA was electrophoresed on a 1% agarose gel containing 2.2 mol/L formaldehyde and 0.02 mol/L 3-(N-morpholino)-propanesulfonic acid (MOPS), pH 7.0, and 1 mmol/L ethylenediaminetetraacetic acid (EDTA). After washing the gel several times in diethylpyrocarbonate (DEPC)-treated water, the RNA was transferred with 10 × standard sodium citrate (SSC) (1.5 mol/L NaCl, 0.15 mol/L sodium citrate) by capillary transfer to a nylon membrane (GeneScreen Plus) (NEN, Boston, MA, USA). A mouse cDNA probe was labeled with [α-^{32}P] deoxycytidine triphosphate (dCTP) (3000 Ci/mmol) (ICN, Irvine, CA, USA) with random prime method (Oligo Labelling Kit) (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). The blot was hybridized overnight at 68°C in Church's buffer containing 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA), 0.5 mol/L $NaHPO_4$, pH 7.2, and 1 mmol/L EDTA. The blot was washed twice for 30 minutes with a solution containing 0.04 mol/L $NaHPO_4$, 5% SDS, 0.5% BSA, and 1 mmol/L EDTA and twice for 30 minutes with a solution of 0.04 mol/L $NaHPO_4$, 1% SDS, and 1 mmol/L EDTA at 68°C. Autoradiography of the blot was performed for 6 hours at –80°C using BioMax MS film and a Transcreen HE intensifying screen (Eastman Kodak, Rochester, NY, USA).

Western blot of mouse kidney for THP

After carbon dioxide narcosis, kidneys were removed from THP wild-type (+/+), heterozygous (+/−), and null (−/−) mice. Each kidney was homogenized with a glass homogenizer at 4°C in 1 mL of lysis buffer containing 25 mmol/L Tris-HCl, pH6.8, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μg/mL pepstatin, 100 μg/mL phenylmethylsulfonyl fluoride (PMSF), and 2 mmol/L EDTA. The homogenates were centrifuged at 5000 × *g* for 20 minutes at 4°C. The supernatants (after filtration through cheesecloth to remove fat) were assayed for total protein with a kit (BCA Protein Assay Kit) (Pierce, Rockford, IL, USA). Samples containing 5 μg of total protein were solubilized in Laemmli buffer containing β-mercaptoethanol and electrophoresed on a 10% SDS-polyacrylamide minigel (Mini-Protean II) (Bio-Rad, Hercules, CA, USA). The proteins were electrophoretically transferred to a nitrocellulose membrane (Nitro Plus, MSI, Westboro, MA, USA). After blocking overnight with 5% nonfat milk in phosphate buffered saline (PBS) at 4°C, the blot was probed with a rabbit antihuman THP antibody (50 μg/mL in PBS) (BTI, Stroughton, MA,USA). The blot was washed three times with PBS/0.05% Tween-20, and probed with a goat antirabbit IgG conjugated with horseradish peroxidase (1:5000) (Sigma Chemical Co., St. Louis, MO, USA). The blot was again washed three times with PBS/0.05% Tween-20. The blotted proteins were detected with a chemiluminescent agent (ECL) (Amersham, Arlington Heights, IL, USA), using BioMax MS film (Eastman Kodak Co.).

Immunohistochemical staining of mouse kidney for THP

Kidneys from $THP^{+/+}$ and $THP^{-/-}$ mice were sectioned sagittally and fixed in 10% buffered formalin. The kidneys were embedded in paraffin and eight micron sections prepared. After deparaffinization in xylene and rehydration through a graded ethanol series and water, the slides were washed in PBS. Endogenous peroxidase was inhibited by incubating the slides for 20 minutes in 0.3% hydrogen peroxide in methanol. After washing in PBS for 20 minutes, nonspecific binding was blocked for 20 minutes with 2% goat serum (Vectastain ABC Kit) (Vector Labs, Burlingame, CA, USA). A rabbit antihuman THP antibody (BTI) diluted 1:3000 in PBS/0.1% BSA was applied to the sections and incubated overnight at 4°C in a humidity chamber. The slides were washed in PBS (10 minutes, twice) and incubated with a secondary biotinylated antibody (goat anti rabbit IgG, Vectastain

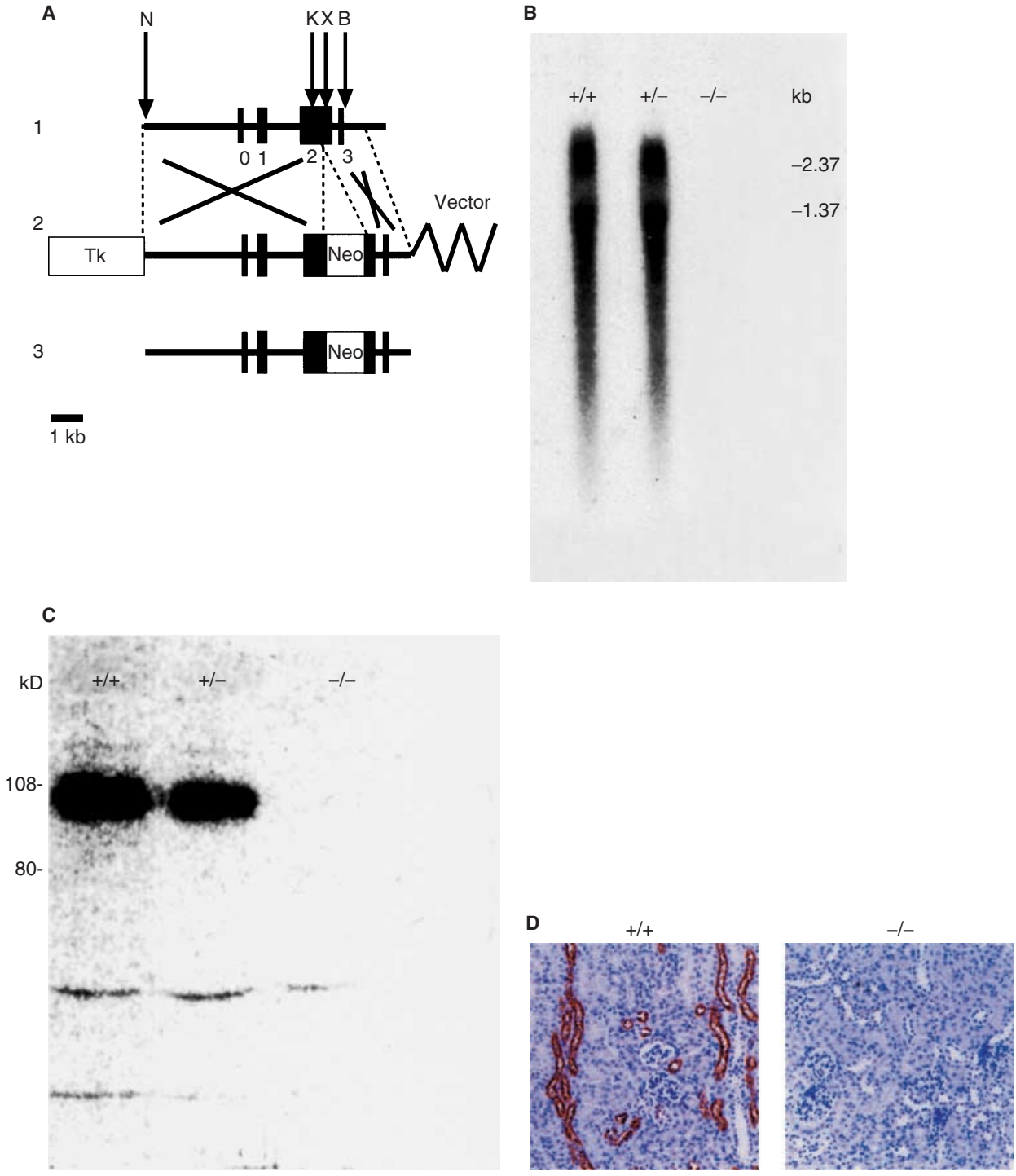

Fig. 1. Strategy for targeted disruption of the mouse Tamm-Horsfall protein (*THP*) gene and confirmation of the absence of THP in *THP* gene knockout mice. (*A*) 1, The *THP* genomic locus consisted of a 6.3 kb Not I (N)-BstXI (B) fragment of the 5′ end of the mouse THP gene which includes exons 0, 1, 2, and 3. 2, The targeting vector consisted of a 2.9 kb Xho I (X)-Not I (N) fragment of a DNA fragment containing the thymidine kinase gene (Tk) that acts as a negative selection marker for random recombinants, a 5.5 kb Not I (N)-Kpn I (K) fragment of the mouse THP gene, an 0.9 kb Kpn I (K)-BstXI (B, converted to a Cla I site) fragment of the mouse THP gene with a 1.6 kb DNA fragment containing the neomycin phosphotransferase gene (Neo) that acts as a positive selection marker for correctly targeted clones, inserted into the Xho I site in exon 2, and a 3 kb Cla I-Xho I fragment of the pGEM7f plasmid (vector). 3, The targeted allele with the "Neo," positive selection cassette, disrupting exon 2 of the mouse THP gene. (*B*) Northern blot of kidney RNA from wild type (+/+), heterozygous (+/–) and null (–/–) mice. (*C*) Western blot of total kidney protein from wild type (+/+), heterozygous (+/–) and null (–/–) mice. (*D*) Immunohistochemical staining of kidney sections from wild type (+/+) and null (–/–) mice.

ABC Kit) at a dilution of 1:200 in PBS/0.1% BSA for 90 minutes at room temperature. The slides were washed in PBS (10 minutes, twice) and the ABC reagent (Vectastain ABC Kit) (Vector Labs) was applied for 30 minutes at room temperature. After washing with PBS, the slides were incubated with a 3,3-diamino benzidine (DAB) solution until brown color developed (monitored through microscope). After placing in tap water, the slides were counterstained with hematoxylin and mounted under cover slips.

Bacterial preparations

E. coli strain J96 (ATCC 700336), a pyelonephritis isolate with capacity to express both type 1 and P fimbriae under specific culture conditions, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and propagated in tryptose broth. An overnight, stationary phase, tryptose broth culture was made from the main culture, and bacteria harvested from the air-broth interface to select for bacteria expressing type 1 fimbriae. The number of bacteria/mL was determined spectrophotometrically at 600 nm. The bacterial suspension was centrifuged at 3600 rpm for 20 minutes, and the pellet washed twice with PBS. A final suspension was made in PBS at 1×10^{12} bacteria/mL. *E. coli* strain UTI89, a clinical cystitis strain mainly expressing type 1 fimbriae, was propagated as above and suspended in PBS at 4×10^{7} bacteria/mL. *E. coli* strain AAEC185/pSH2, a recombinant strain with full type 1 pilus operon, expressing only type 1 fimbriae [24], was propagated in tryptose broth with chloramphenicol, centrifuged, and resuspended in PBS at 4×10^{11} bacteria/mL. *E. coli* strain DS17, a pyelonephritis isolate expressing mainly P fimbriae, was propagated in Luria-Bertani (LB) medium, centrifuged, and resuspended in PBS at 1×10^{11} bacteria/mL.

Animal experimental procedures

Bacteriuria studies. $THP^{+/+}$ and $THP^{-/-}$ female mice were kept in separate mouse cages and allowed to eat and drink ad libitum. The perineum was cleaned with 70% isopropanol. Gentle pressure was applied to the suprapubic region of the mouse, and the urine drops appearing at the urethral meatus aspirated with a sterile pipet. Baseline urine specimens were obtained and cultured on Eosin Methylene Blue (EMB) agar (Becton Dickinson, Sparks, MD, USA) at 37°C overnight to exclude preexisting infection. A few mice were excluded from experiments due to preexisting infection. Spontaneous urinary tract infections were rare and did not occur more frequently in the $THP^{-/-}$ mice in comparison with $THP^{+/+}$ mice. The mice were deprived of water for 4 hours and anesthetized with xylazine (Butler, Columbus, OH, USA), 8mg/kg, and ketamine (Fort Dodge Animal Health, Fort Dodge, IA, USA), 120 mg/kg. Preexisting urine was removed from the bladder by gentle squeezing before inoculation. Under aseptic conditions, 10 μL of a 1×10^{12} bacteria/mL suspension of *E. coli* strain J96 was introduced into the bladder transurethrally using a 50 μL syringe (Hamilton, Reno, NV, USA) fitted with a 28 G needle and polyethylene tubing with an inner diameter of 0.28 mm and outer diameter of 0.61 mm (Becton Dickinson). The mice were allowed to recover from anesthesia and allowed free access to food and water. In experiments with *E. coli* strain UTI89, 25 μL of a 4×10^{7} bacteria/mL suspension was introduced into the bladder and the catheter clamped. After 1 hour, the clamp was released and the catheter removed. In experiments with *E. coli* strain AAEC185/pSH2, 25 μL of a 4×10^{11} bacteria/mL suspension was introduced transurethrally using the same methods as for UTI89. For bacteriuria studies, mouse urine was collected aseptically at 1, 3, 5, and 7 days post-inoculation and weekly thereafter, until the urine cultures became negative. The urine specimens were serially diluted and cultured on EMB agar overnight at 37°C. Colony forming units (CFU) were counted the next day and the number of *E. coli*/mL of urine was calculated and expressed as CFU/mL of urine.

Bladder colonization studies. A suspension of *E. coli* strain J96 was introduced into mouse bladders as above. Mice were euthanized 24 hours after transurethral inoculation of *E. coli*, using carbon dioxide narcosis. The urine was expressed from the bladder prior to euthanasia. The bladders were removed aseptically and bisected. Half of each bladder was weighed and homogenized separately using glass tissue grinders (Kontes, Vineland, NJ, USA). The homogenates were serially diluted in PBS and cultured on EMB agar. On the next day the CFUs on each EMB plate were counted and expressed as CFU/mg bladder tissue. The other half of each bladder was processed for histopathologic examination. Bladder tissue specimens were fixed overnight in 10% neutral buffered formalin. Sections of 5 μm thickness were cut from paraffin-embedded tissue and stained with hematoxylin and eosin. The slides were examined by light microscopy by a pathologist.

Kidney colonization studies. In experiments with *E. coli* strain DS17, a pyelonephritis isolate expressing mainly P fimbriae, 100 μL of a 1×10^{11} bacteria/mL suspension was inoculated into mice transurethrally using a 100 μL syringe (Hamilton) fitted with a 28 G needle and polyethylene tubing. The catheter attached to the syringe was allowed to remain in the urethra for 1 hour. Mice were euthanized 5 days after transurethral inoculation of *E. coli*, using carbon dioxide narcosis. The urine was expressed from the bladder prior to euthanasia. The kidneys were removed aseptically, weighed and homogenized separately using glass tissue grinders (Kontes). The homogenates were serially diluted in PBS and cultured

Table 1. Bacteria/mL of urine in Tamm-Horsfall protein (THP)$^{+/+}$ and THP$^{-/-}$ mice after inoculation of *Escherichia coli* strain J96

Experiment Number	Day-3		Day-5		Day-14	
	THP$^{+/+}$	THP$^{-/-}$	THP$^{+/+}$	THP$^{-/-}$	THP$^{+/+}$	THP$^{-/-}$
1	0	1.4×10^4	0	0	0	0
2	0	2×10^3	0	0	0	0
3	1×10^3	$>10^{12}$	0	1.1×10^{10}	0	Died
4	8.8×10^2	8.1×10^{10}	6.4×10^2	0.3×10^{10}	0	Died
5	0	8×10^2	0	0	0	0
6	0	2.2×10^3	0	0	0	0
7	7.4×10^2	4.5×10^4	0	1×10^2	0	0

on EMB agar. On the next day the CFUs on each EMB plate were counted and expressed as CFU/mg kidney tissue.

The animal experiments were pre approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Oklahoma Health Sciences Center.

Statistical analysis

Data are expressed as means ± SE. Student *t* test was used for comparison of groups using the SPSS statistical program (SPSS, Inc., Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

RESULTS

Bacteriuria studies

Results from seven separate bacteriuria experiments are shown in Table 1. In each experiment with *E. coli* strain J96, the THP$^{-/-}$ mouse cleared the bacteria from urine slower than the THP$^{+/+}$ mouse. On day 3, urine from four out of seven THP$^{+/+}$ mice was clear of bacteria, while seven out of seven THP$^{-/-}$ mice showed bacteriuria. On day 5, six out of seven THP$^{+/+}$ mice had cleared the bacteria from the urine while three out of seven THP$^{-/-}$ continued to excrete bacteria. By day 14, all THP$^{+/+}$ mice had cleared the bacteria from urine, while five out of seven THP$^{-/-}$ mice had cleared the bacteria from urine. Two out of seven THP$^{-/-}$ mice died by day 14, and their kidneys showed severe pyelonephritis and micro-abscesses in the kidney.

Similar experiments were performed with *E. coli* strains UTI89 and AAEC185/pSH2. In studies with *E. coli* strain UTI89 (Fig. 2A), the THP$^{-/-}$ mice had more severe bacteriuria at 24 hours post-inoculation (mean $\log_{10}$ CFU/mL + 1, 6.84 ± 0.32) compared to the THP$^{+/+}$ mice (mean $\log_{10}$ CFU/mL + 1, 3.70 ± 0.91; $P = 0.004$). Experiments with *E. coli* strain AAEC185/pSH2 (Fig. 2B) also showed a greater number of *E. coli*/mL of urine at 24 hours post-inoculation in THP$^{-/-}$ mice (mean $\log_{10}$ CFU/mL 5.6 ± 0.43) compared to THP$^{+/+}$ mice (mean $\log_{10}$ CFU/mL 3.66 ± 0.36; $P = 0.004$) in five experiments.

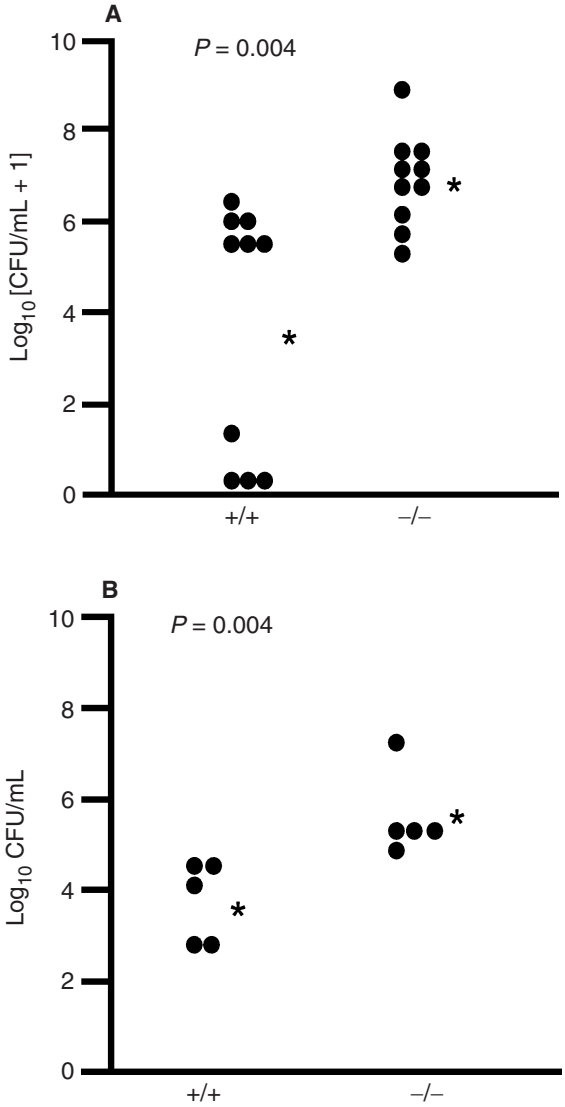

Fig. 2. Distribution of colony forming units (CFU)/mL of urine 24 hours after transurethral inoculation of *Escherichia coli.* (*A*) UTI89 (expressed as the $\log_{10}$ CFU/mL + 1) in wild-type (+/+) and null (–/–) mice. (*B*) AAEC185/pSH2 (expressed as the $\log_{10}$ CFU/mL) in wild-type (+/+) and null (–/–) mice.

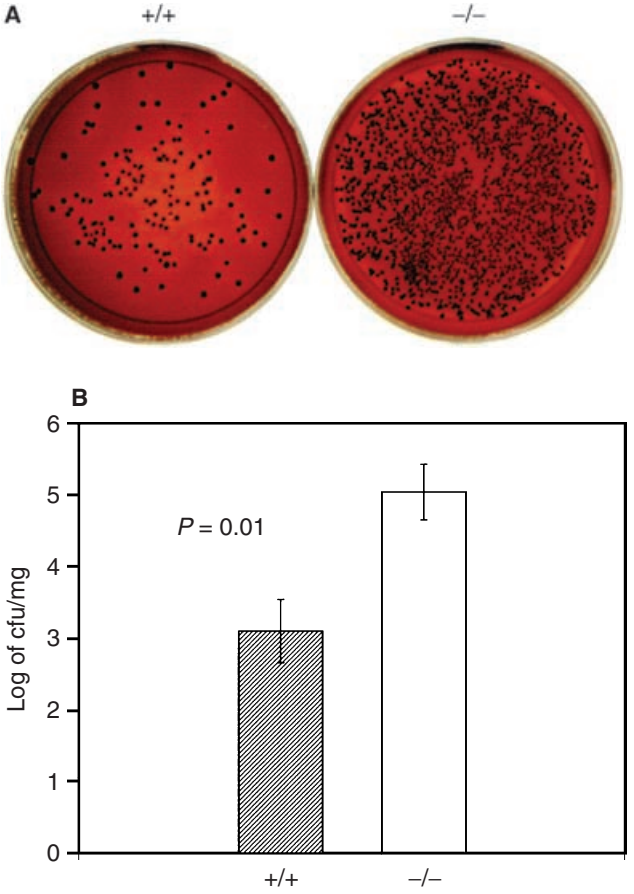

Fig. 3. Bacterial colonies isolated from homogenized bladder tissue 24 hours after transurethral inoculation of *Escherichia coli* strain J96. (*A*) The Eosin Methylene Blue agar plates show bacterial colonies isolated from homogenized bladder tissue of wild type (+/+) and null (–/–) mice 24 hours after transurethral inoculation with *E. coli* J96. (*B*) Comparison of colony forming units (CFU)/mg of bladder tissue (expressed as $\log_{10}$ CFU/mg tissue) 24 hours after transurethral inoculation of wild type (+/+) and null (–/–) mice with *E. coli* J96.

Bladder colonization studies

Bladder tissue homogenates were cultured after transurethral inoculation of *E. coli strain* J96 and bacterial load/mg of tissue demonstrated that $THP^{-/-}$ mice had more severe colonization than $THP^{+/+}$ mice (mean $\log_{10}$ CFU/mg 5.07 ± 0.38 in $THP^{-/-}$ mice versus mean $\log_{10}$ CFU/mg 3.15 ± 0.45 in $THP^{+/+}$ mice, $P = 0.01$) in five consecutive experiments (Fig. 3). Bladder tissues were examined for histologic changes and there were no differences in $THP^{+/+}$ and $THP^{-/-}$ mice bladders at 24 hours after inoculation.

Kidney colonization studies

Kidney tissue homogenates were cultured 5 days after transurethral inoculation of the P fimbriated *E. coli* strain DS17. Bacterial load/mg of tissue demonstrated no significant difference between the $THP^{-/-}$ mice and the $THP^{+/+}$ mice in six experiments (Fig. 4).

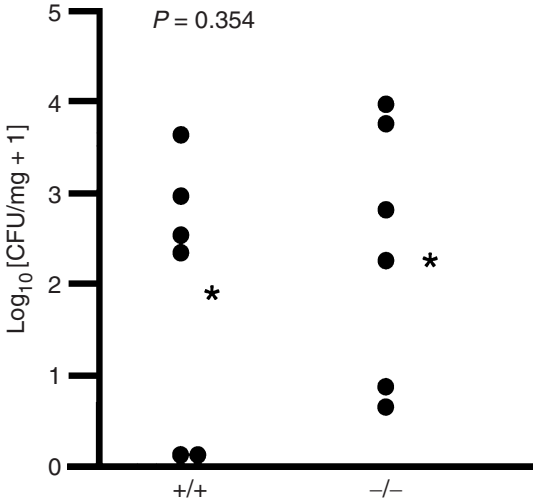

Fig. 4. Distribution of colony forming units (CFU)/mg of kidney tissue 5 days after transurethral inoculation of *Escherichia coli* DS 17 (expressed as the $\log_{10}$ CFU/mg +1) in wild-type (+/+) and null (–/–) mice.

DISCUSSION

The biologic function of THP (also known as uromucoid [25] or uromodulin [26]) has been speculated upon since 1895. Several physiologic and pathologic roles have been postulated for THP such as ion transport in kidney tubules, renal excretion of cytokines, and the pathogenesis of kidney stones, and of acute renal failure. Its exact biologic function, however, has remained unclear [3–6]. Orskov, Orskov, and Birch-Anderson [15] and Orskov, Ferencz, and Orskov [16] first reported that THP in solution could bind *E. coli*, and that this binding was inhibited by D-mannose. Subsequently, THP was shown to bind bacteria on solid surfaces [17, 18] and to the surface of bacteria by electron microscopy [27]. THP has also been shown to inhibit binding of bacteria to human kidney cells in tissue culture [28] and to synthetic analogues of urothelial surface receptors [29]. The biologic significance of these in vitro findings, however, has remained unclear thus far. Our data provide new, in vivo, evidence that interactions between THP and *E. coli* are likely to be biologically important.

E. coli are the most common cause of urinary tract infection. Binding of *E. coli* to the urothelium is critically dependent upon the type of fimbriae present on the bacterial cell surface. Type 1 fimbriae bind mannose residues on the cell surface and P fimbriae bind a globoside that consists of the tetrasaccharide GalNAcβ1–3Galα1–4Galβ1–4Glc linked to ceramide [30]. In this study, we found that $THP^{-/-}$ mice are more prone to infection by type 1 but not P fimbriated *E. coli*. This finding is consistent with the

fact that THP contains high mannose structures among its oligosaccharides but not the ligand for P fimbriae. The function of the diverse array of complex type carbohydrate structures on the surface of THP remains uncertain at this time. It is likely that they serve as binding sites for other substances that need to be cleared from the urine such as cells, cytokines, crystals, ions, and other microorganisms.

Overall, these data provide the first direct in vivo evidence that THP serves a role in continually clearing the urine of contaminating bacteria. Not only do these findings identify a novel host defense mechanism against urinary tract infection, they also assign an important biologic function to urine's most abundant protein.

Recently, point mutations in the human THP gene have been shown to be associated with medullary cystic disease of kidneys [31]. No cysts or fibrosis were observed in $THP^{-/-}$ mice in our experiments up to 24 months.

NOTE ADDED IN PROOF

This article was presented, in parts, in abstract form at the American Society of Nephrology meetings in November 2002 and 2003. Another study, submitted after the acceptance of this article, has independently confirmed the findings contained in this article (MO L et al: Ablation of the structural gene for Tamm-Horsfall protein increases the susceptibility of mice to bladder colonization by type 1-fimbriated *Escherichia coli.* Am J Physiol. In press.).

Reprint requests to Satish Kumar, M.D., Department of Medicine, Nephrology Section, University of Oklahoma Health Sciences Center, 920 S. L. Young Blvd., WP2250 Oklahoma City, OK 73104.
E-mail: satish-kumar@ouhsc.edu

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